

## PLANT ADAPTATION TO COLD

## I. CHLOROPHYLL

## II. MINERALS

by

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A thesis submitted in partial fulfillment  
of the requirements for the degree


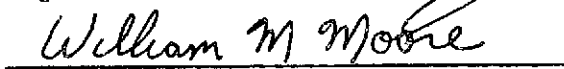
of

MASTER OF SCIENCE

in

Department of Plant Science  
(Plant Ecology)

Approved:

  
Major Professor  
Committee Member  
Committee Member  
Dean of Graduate Studies(NASA-CR-139528) PLANT ADAPTATION TO  
COLD. 1: CHLOROPHYLL. 2: MINERALS  
M.S. Thesis (Utah State Univ.) 80 P HC  
\$7.00

79 CSCL 06C

G3/04

Unclas  
46400

N74-30481

UTAH STATE UNIVERSITY  
Logan, Utah

1972

#### ACKNOWLEDGMENTS

I would like to extend my gratitude to Dr. Frank B. Salisbury who served as my major professor during the course of this study, and to Dr. Martyn M. Caldwell and Dr. William M. Moore who were members of my supervisory committee.

I would also like to thank Bryce Bennett, Dr. Alice Denney, Dr. David Gibby, Dr. Steven Kimball, and Dr. Manfred Weidner for their discussion and encouragement. I am especially grateful to Dr. Weidner for his help in setting up the tunnel facility in Franklin Basin, and to Dr. Kimball for his aid in preparing the manuscript for this thesis.

My sincere appreciation goes to the National Aeronautics and Space Administration for the financial support which made this study possible.



Peter Rosen

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# ABSTRACT

## Plant Adaptation to Cold

### I. Chlorophyll

### II. Minerals

by

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Utah State University, 1972

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Department: Plant Science

A number of montane herbs in northern Utah typically form flower buds beneath the snow cover and flower either through it or immediately after its recession. Two of these species, one naturally occurring, Claytonia lanceolata, and one cultivated bulb, Galanthus nivalis, were investigated for their response to this stress environment.

Snow depth patterns, chlorophyll content of tissues, and plants grown in light-tight boxes, suggest that light passing through the snow to reach plants growing underneath is not critically involved in the timing of their developmental cycles or in their ability to endure this low temperature environment.

Ability to endure stress seems to be closely related in a number of ways to activity at the plant membranes. Plants were protected from low temperature damage by application of cytokinin or calcium, both of which probably acted at the membrane. Potassium calcium antagonisms were reflected in the internal distribution of the ions under natural

stress conditions; and plants that differentiated at the meristem while growing through the snow accumulated calcium at the tip during this growth.

(80 pages)



## INTRODUCTION

Plants that are adapted to survive in low temperature environments may do so in a number of ways. One example of a broad distinction in this area would be the scheme of hardiness vs. resistance as outlined by Levitt (1967). This thesis deals with a similar distinction between resistance and endurance. Resistance is designated as the property of avoiding low temperatures in growing plant tissues. Endurance is designated as the property of avoiding the damage caused by these low temperatures when they do occur.

Growth under snow is a form of resistance since it insulates the plant. Other means of resistance may include changes in life cycle timing to accomodate a dormant state during cold periods. While plant temperatures will drop in this last case, the plant will be better able to survive if growth is not occurring during this time.

Mechanisms of endurance will allow the plant to grow despite the debilitating effects of intracellular ice formation, dehydration of the protoplasm, and configuration changes of important molecules. They must not only prevent structural damage but also allow normal function. The plants chosen for this study should provide examples of these mechanisms because they grow at 0 C. Although they will not experience the debilitating effects to the extent of plants which remain dormant at lower temperatures, their strategies for maintaining growth under these conditions should include endurance mechanisms.

Membranes are susceptible to low temperature damage in both structural and functional ways. The molecular structure of the membrane

is stressed by low temperatures, and the formation of extracellular ice stresses the permeability activities. Research indicates that membranes, particularly in the chloroplasts and mitochondria, may be the weakest point in a plant stressed by cold (Mazur, 1969). This research entails some investigation into mechanisms of endurance at this point. They are additionally important because membranes may be the weak point in many kinds of stress situations (e.g., herbicides, water stress, and insect damage) in addition to cold. The mechanisms for dealing with these problems may have a wide variety of applications in plant ecology.

## REVIEW OF LITERATURE

### Growth under Snow

Environmental influences on plant growth under the snow may be divided into two major types: the continuous effects of light, temperature, and moisture on plant metabolism; and the discrete influences on plant development that may occur at some point in the developmental cycle of those plants that initiate growth under the snow. This second kind of influence may be negligible if timing of spring growth is determined primarily by temperature patterns of the previous Fall, as suggested by Goryshina (1965). The question remains whether adaptation to low temperature stress in these plants includes some adaptation to facets of the environment under the snow other than low temperature, i.e., mechanisms of resistance unique to the environment beneath the snow pack.

The preparations for photosynthesis by chlorophyll production has been an attractive possibility for a metabolic initiator reaction in plants overwintering in cold environments. Observations have indicated that spring ephemerals that grow at near-freezing temperatures under snow cover may be able to synthesize chlorophyll with only the light available to them beneath the snowpack (Kimball, 1969). This synthesis may be correlated with an early or accelerated release from winter dormancy through the overall patterns of light, temperature, moisture, and carbohydrate metabolism. Current literature is inconclusive, however, on the existence of such adaptations.

The carbohydrate metabolism of plants growing under extreme cold conditions shows distinct adaptation to the environment, pointing up the possibility that high sugars are beneficial to the over-wintering plants as an osmotic protector agent (Fonda and Bliss, 1966; Risser and Cottam, 1967). While this protection may or may not be sufficient to account for the maintenance of tissue viability at low temperatures (it probably is not), it is unclear whether this phenomenon is connected to the stimulation of active growth in the spring. The correlation of changing carbohydrate patterns and plant growth, while not a fool-proof way of determining cause and effect relationships between these two processes, would seem to indicate that carbohydrate changes are effects, not causes, of changing growth patterns (Mooney and Billings, 1960). The connection of these patterns to light penetrating the snow seems unlikely after consideration of the results of Struik (1965), who used plants growing without snow cover and found similar cycles. Burt (1966), however, indicates that "sink strength," the ability to store and utilize the products of photosynthesis, is dependent on temperature changes, including those under the snowpack. Veniza and Grandtner (1965) suggest that these patterns may be sensitive to small radiation changes, which in their study were due to the changing canopy but could also be due to the melting snowpack.

### Light

It is tempting to speculate that plants undergoing part of their life cycles under snow cover (e.g., Ranunculus sp., Erythronium sp., Claytonia sp., and Orogenia linearifolia) are adapted so that light penetrating the snow plays some part in their development. Possible

effects of this light include the conversion of protochlorophyll to chlorophyll--a light requiring process in most angiosperms (Boardman, 1966)--acting with a change in heat and carbon dioxide concentration in the immediate environment of the plant to trigger anabolism. Protochlorophyll could be converted to chlorophyll at the light levels typically existing under the snow about 1 to 2 weeks before total melt in the Wasatch Mountains (Pearson and Dinsdale, 1963). The pathways that exist in gymnosperms allow for conversion in the dark (Boardman, 1966). Although these processes are temperature dependent, they are not inhibited at 0 C (Virgin, 1955). Photochemical pathways may also be active at very low light intensities. They demonstrate a possible quantum yield of 1 and efficiency of 0.6 (Smith, 1958). This conversion occurs in vivo as a natural precursor to photosynthesis (Zeldin and Radke, 1971). But it may need to be accompanied by active photosynthesis since the turnover time of the enzyme that produces protochlorophyll has been shown to be about 10 minutes in some systems, with production dependent on the usage of chlorophyllide (Suzer and Sauer, 1971).

Light is probably not involved in the resistance of plants growing under snow to cold temperature, however. Research shows normal etiolation syndromes for plants grown under black plastic and snow cover (Bennett, 1971). Furthermore, it is virtually impossible to conceive of any kind of early plant warming under a snow pack, which would act as a huge sink for any heat evolved. The period between snow depth low enough for light transmission and total melt is also quite short. This raises the question of whether that period of time would be a credible influence on development. Billings and Mooney (1968) have

indicated that time of release from snow cover is compensated for by a telescoping of phenological events in plants released later in the season. In any case adaptations to a shortened growing season are distinct from adaptations to low temperatures. Literature concerning alpine environments does not indicate substantial effects of light transmission through the snow (Morris, 1965; Obreska, 1969; Grew, 1966; Houvillat, 1970; Marr, 1967; Marcus, 1969).

In extreme ecological conditions, however, light reception might affect plant distribution and thus the ability to live in environments of colder mean temperatures. Altitude, latitude, and microclimate will affect this system. In the colder temperate regions, gentle south facing slopes would provide the best light environment in the Northern Hemisphere, while at lower latitudes, slope and aspect would become less important (Lee and Baumgartner, 1966; Terjung et al., 1969).

Light transmission through snow most closely follows the equation:

$$I_x = I_0 (e^{-vx}),$$

where  $I_x$  is the radiation at  $x$  centimeters,  $I_0$  is the radiation at the surface, and  $v$  is the extinction coefficient of the snow. Extinction coefficients vary widely according to snow quality (i.e., ice layers, snow compaction, structure, and water content). In powdery snow no systematic relation exists between transmissivity and wavelength, but as water content increases, generally, extinction coefficients increase with wavelength (Geiger, 1965). Late in the season, therefore, as the snow compacts, we may approach a relation between wavelength and transmissivity as shown in Figure 1. In the line labeled "idealized-high water content." Studies of transmissivity in the

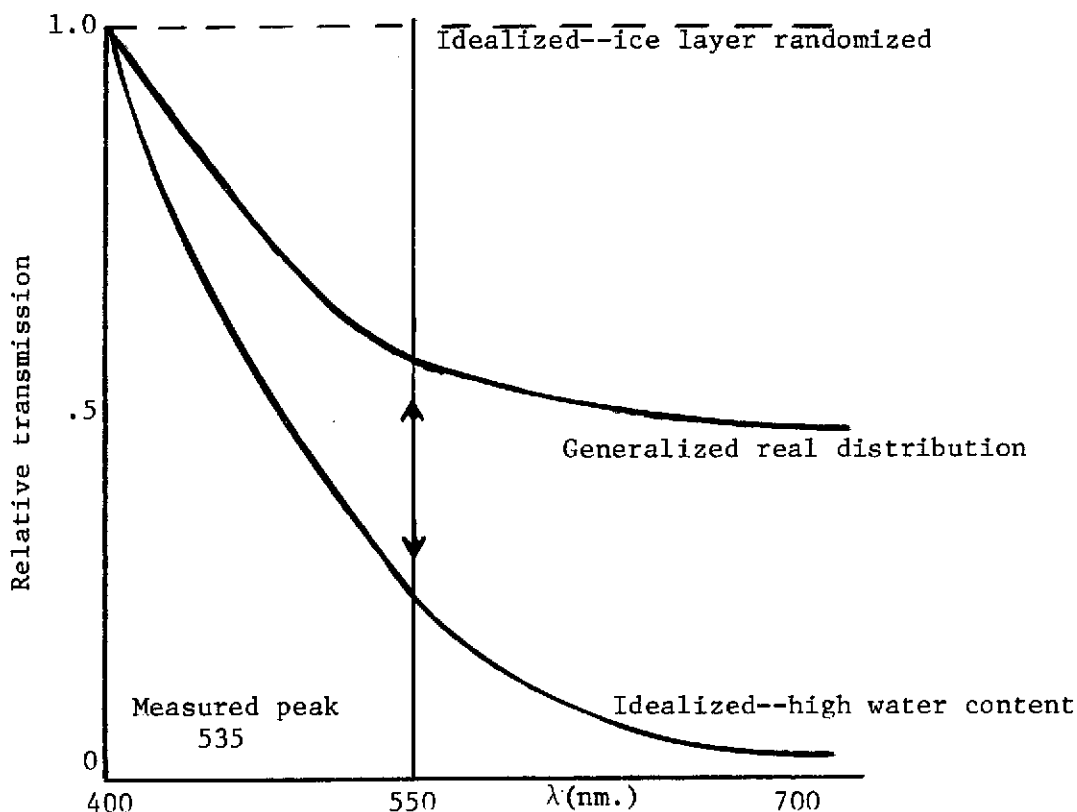


Figure 1. Light transmission through snow pack.

Wasatch Mountains showed that a peak actually existed around 550 nm., with transmission dropping off again at shorter wavelengths (Bennett, 1971).

Ice transmits more short wavelength radiation than snow; and the formation of ice layers in the snow pack, which normally occurs during this period of its development, would have a randomizing effect on light transmission, approaching the line so labeled in Figure 1 (Lyubomirova, 1963). The curve labeled "generalized real distribution" describes an idealization of possible wavelength distribution in light reaching the plant, neglecting the influence of solar spectra.

### Temperature and carbon dioxide

Kimball (1969) has observed that in the Wasatch Mountains light transmitted through snow may warm plants to the point where they melt a portion of the snow immediately surrounding them. This separation of plant and snow would eliminate conductive transfer between plant and snow, reducing heat transfer to mostly radiative means (convection would be minimal under these conditions). Feedback effect of plant cover on snow melt which may be attributable to plant warming have been observed (Monteith, 1956).

Along with temperature, carbon dioxide concentration will affect the development of plants under the snow. Carbon dioxide produced by soil microorganisms may accumulate if diffusion outwards is blocked by an impermeable ice layer. Even without this ice layer, carbon dioxide might build up due to the increasing density and ice content of the snow pack. Increasing density will decrease pore space for outward diffusion, and increasing ice content will decrease carbon dioxide absorption by the snow (Essery and Gane, 1952). This carbon dioxide would escape as decreasing snow depth reduced the diffusion barrier.

The thermal conductivity of any portion of the snow pack in calories  $\text{cm}^{-1} \text{ sec}^{-1} \text{ deg}^{-1}$  is proportional to the square of the density of the snow. Radiation penetration and density profiles in many snow packs during mid-season put the temperature maximum somewhere below the top of the pack (Geiger, 1965). While this relationship may be traced to the structure of individual snow particles (Nakaya, 1954) and the drifting characteristics of loose snow (Mellor and Radok, 1960), these effects will be negligible compared to the general pattern of ice and water layers. Ice layers may form from alternate freezing and thawing



at the surface of the snow or internally at the temperature maximums described above. In addition to decreasing carbon dioxide diffusion, these ice layers, and the bands of water accumulated above them, will resist heat reradiation out of the pack during the night (Geiger, 1965).

### Stress Protection

#### Specific responses

Environmental factors are related to endurance only inasmuch as they involve activity at the site of low temperature damage. Light, carbon dioxide, and temperature under the snow pack may be related to resistance mechanisms to the extent that they regulate life cycle timing, but there is no evidence for their involvement in mechanisms of endurance. Endurance mechanisms must be active at the heart of the low temperature destruction processes; the point where not only plant function, but structure also, is affected.

Existing evidence indicates that membranes are this primary target (Heber, 1967; Mazur, 1969 and 1970). Evidence indicates that membrane deterioration is part of a specific syndrome that constitutes plant response to all kinds of environmental stresses including not only low temperature, but herbicides, water stress, and insect damage, as well (Campbell, 1971). The associated events (i.e., destruction of outer chloroplast membranes, loss of internal chloroplast structure, and overall loss of membrane structure throughout the cell) form the "action" syndrome of the stress response. One aspect of the "reaction" syndrome, increasing concentrations of endoplasmic reticulum, particularly becoming oriented parallel to the nuclear envelope, has also been observed with the electron microscope. Though the stepwise nature of these changes is

not clear, serial effects have been noted in treatments of increasing cold stress in the order of action events indicated above (Kimball, 1971), as well as under a variety of temperature and light regimes, including high temperature stress environments (Daniell, Chappell, and Couch, 1969; Taylor and Craig, 1971). Experiments using isolated chloroplasts have demonstrated the chloroplast specificity of this response and elucidated some of the details involving mineral ions suggesting their direct involvement (Nishida, Tamai, and Umemoto, 1966; Shakov and Golubkova, 1960).

While one extreme of this membrane response might be configurational change of lipid components in the lipo-protein matrices, membrane susceptibility could arise from other, more complex, considerations. Outer membranes are stressed by the unbalanced water potentials generated across them by the formation of intracellular ice. Chemical imbalance across a membrane could have severe destructive effects. The chemical transfer processes necessary for normal energy flow according to Mitchell's theory of chemiosmosis are particularly susceptible in chloroplasts and mitochondria (Packer, Murakami, and Mehard, 1970). Mitochondria are especially sensitive to ion imbalances because they are the site of ATP production. These imbalances may arise from increasing concentrations of ions due to ice formation. The particular susceptibility of chloroplasts to stress is less clear. It may arise from their obligatory reception of radiant energy from the environment regardless of how operable other metabolic pathways may be for utilization of this energy. In stress situations these pathways may not be operative, and the forced accumulation of this energy could have destructive effects (Kimball, personal communication).

### General syndrome

The importance of being able to view plant responses to cold temperature as part of an overall general stress response syndrome are evident if we look at what has been done with a similar approach to animal stress. Selye (1956) noted that sickness in animals was accompanied by a chain of events that was associated not with any particular disease, but with the simple fact of being sick or of being stressed in other ways. This syndrome included enlargement of the adrenal gland, the thymus, and the spleen, shrinkage of the lymph nodes, and ulceration of the stomach. Investigation of this phenomenon led him to the elucidation of the hormone system that mediates these responses, and has wide application to the understanding of many problems in animal ecology (e.g., overpopulation stress feedbacks) and in the applied aspects of these problems in the field of medicine.

Figure 2, from Selye (1956), describes an experiment in which rats are exposed to cold temperature stress for varying periods of time

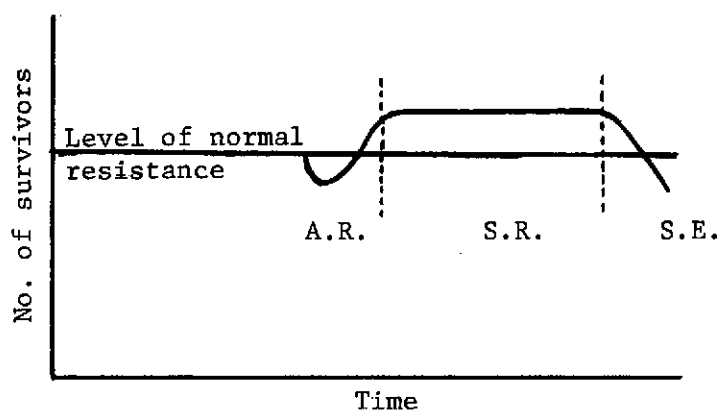


Figure 2. Stress response phases.

and then transferred to another cold environment, where the number of survivors is compared to the length of time in the original stress conditions. After an initial period of time for adaptation (A.R. = adaptation reaction, a phase which Selye characterizes as requiring the expenditure of "adaptation energy"), there is a period of time that Selye designates as the "stage of resistance" (S.R. = a period during which resistance is higher than normal; S.E. = stage of exhaustion, when the original stress is debilitating). The S.R. is strongly analagous to hardening phenomena in plants. Stocker (1960), in investigating this stage of plants, which he calls the "restitution phase," demonstrates a correlation between this resistance and a strengthening of the protoplasmic matrix. Stocker also characterizes a "reaction phase," similar to Selye's "adaptation reaction," and demonstrates these phases by measurement of a variety of physiological functions, e.g., photosynthesis and respiration, in plants exposed to water stress. Protoplasmic viscosity, discussed as a prime mediator of these reactions, is again connected to the actions of mineral ions.

Hormone action in Selye's stress system involves ACTH and the adrenal cortical hormone, cortisone. The dynamic balance of these hormones determines the extent of the destruction of the organism. While cortisone is in some cases a general reliever of stress irritations, certain stress situations may be damaging through a requirement for excessive amounts of cortisone that disrupts the normal balance of the hormone system. The system relies on a balance of two hormones that have opposite effects on the inflammations that are the local symptoms of stress. While inflammation may be destructive in itself, it also prevents the spread of pathogens to other parts of the organism by

creating impassable walls. The balance between preventing destructive inflammation and maintaining the local stress imbalance in a limited area is the physiological problem encountered by the stress response hormones.

#### Cytokinin involvement

In speculating about what kind of hormone system, if any, might be operative in plants, our attention is drawn to cytokinin. Its action as a chlorophyll protector in senescing tissue (Leopold and Kawase, 1964; Osborne and Moss, 1963), if related to the fact that the plant stress response is largely a chlorophyll destruction syndrome, is highly suggestive. The efficacy of cytokinin as a "phytoaspirin" (Lang, 1967), i.e., a protective agent in a wide variety of environmental stresses, is further indicative of its possible role. Effects of cytokinin in promoting frost resistance are documented in six vegetable species (Kuraishi, 1968). There is some indirect indication that chloroplast protection in vivo involves a cytokinin reaction. Root chilling alone (the site of cytokinin biosynthesis--Skoog and Armstrong, 1970) affects leaf chloroplast behavior (Shakov and Golubkova, 1960; Taylor and Rowley, 1971; Spomer and Salisbury, 1968); but this, of course, could have countless other explanations.

Possible mechanisms of cytokinin protection lie in the increase in protein synthesis caused by cytokinins (Osborne, 1962); which could be a repair response to replace lost membrane protein, or a stimulation of osmotic protection by increase in soluble protein (Pauli and Zech, 1964; Barnett and Naylor, 1966; Gerloff, Stallman, and Smith, 1967; Stewart and Guinn, 1971; Heber, 1967, 1970; Jung, Shih, and

Shelton, 1967; Siminovitch, Gfeller, and Rheaume, 1967). Additional evidence indicates that cytokinin may directly protect proteins from destruction (Kuraishi, 1968) and increase free sugar pools (Berridge and Ralph, 1971) for mobilization for lipid synthesis. Lipid synthesis has also been suggested as a direct, ATP mediated, cytokinin response (Shaefer, Sharp, and St. John, 1971) and could have an important repair function in membrane damage responses. This kind of protection has been studied on the basis of saturated-unsaturated lipid ratios in plant membranes, and their relation to frost hardness (Kuiper, 1970). It could be related to the extreme cases of low temperature damage in the cases where lipid solubility is affected by the cold.

### Minerals

#### General role of ions in metabolism

Although there is strong indication of the involvement of cytokinin in plant endurance of low temperature stress, presently we can only speculate as to the possible mechanisms of this involvement. No evidence exists that cytokinin is acting directly at the membrane. The involvement of mineral ions at the membrane is clearly indicated. The study of the mineral characteristics of plant growth under conditions of stress, however, requires first some understanding of the complexities of mineral relations under normal conditions.

The abundance of functional and uptake interactions among the various minerals has been widely studied, but the data represent a mass of uncoordinated and often contradictory conclusions. It is important, however, that we look at the basic concepts and trends in the interactions of the ions with which we will be concerned.

Apart from the effects on protoplasmic composition and membrane integrity, mineral ions may affect the viability of the plant through their action as enzyme cofactors. Bygrave (1967) discussed the large number of ways by which this might occur. Of the ions we will be dealing with, potassium is a known cofactor for pyruvate kinase (see Sugiyama, Gota, and Akazawa, 1968), and Bygrave further suggests that the divalent ions, calcium and magnesium, are antagonistically involved in this process. Sodium/potassium ratios are known to control ATPase formation in some plants (Kylin and Gee, 1970), as well as in animals, and Bygrave indicated that calcium may inhibit this effect by competing with magnesium associated ATP. Potassium and magnesium are thought to be required ions for all phosphate transfer reactions in the cell, and the calcium/magnesium antagonism in this competition has been observed in isolated mitochondria (Raaflaub, 1953). It appears then, that these four ions could have a powerful regulatory effect on the plant, particularly through glycolysis.

The effects of these processes on plant viability itself, have commonly been studied with potassium as the major ion. However, Macovschi and Marineanu (1967, 1968) have determined that the relative amounts of potassium, calcium, and sodium in corn seedlings have an important effect on what they call "biostructure," a property very similar to Stocker's protoplasmic viscosity. Normal variation in ion balances during development should reflect this metabolic control, unless the amounts available were in excess of those required.

Sparks (1969) studied potassium movements during development in non-fruiting pecan trees and showed that, while the absolute quantity of potassium increased during active growth, it may decrease in percent

in the plant, since the dry weight increase of the whole plant was even greater. Greatest accumulation of potassium was in the leaves and catkins, with the branches accumulating the least. Absolute amounts by weight, however, were greatest in the leaves, then the branches, catkins, and shoots. Potassium loss after active growth came first in the branches, then the shoots, and then the leaves. This occurred even in these non-fruiting trees, though its occurrence in fruiting trees has been attributed to transport of potassium from the leaves and shoots into the fruit. Potassium was lower, however, in the leaves of fruiting shoots, even on one tree containing both fruiting and non-fruiting shoots (Hunter and Hammar, 1957).

Davies and Winsor (1969) have shown that potassium will influence the fruit composition of the tomato plant in a number of ways, particularly acidity, while other ions (e.g., magnesium) do not. In a four-year experiment with coffee plants, Robinson (1969) found a significant shift in the balances of potassium, calcium, and magnesium with season. He particularly noted that in nearly mature plants that had suffered dry weather (which shifts the balances) and then received a short rain, potassium falls off and calcium rises. This is similar to the shifts found in the data for this thesis, in plants beginning active growth at low temperatures.

It is important when studying the effects of these ions on growth to try to separate the functional or internal effects on metabolism from the external uptake interactions. These external interactions may include substitutions, competitions, or promotions. Any consideration of these processes must take into account the work of Epstein (1966) on the "dual mechanisms" of ion absorption, which showed that ion



transport across the outer root membranes depends on two active systems operable at high and low concentration ranges, perhaps depending on two kinds of carrier proteins.

Johansen, Edwards, and Longeragen (1968), however, working with barley seedlings, suggest that calcium may not have a low concentration mechanism separate from the high concentration carrier. Potassium, they found, will greatly depress calcium uptake, and thereby the internal concentration, when applied in 200  $\mu\text{M}$  quantities. This effect is as strong when the concentration of calcium is 2500  $\mu\text{M}$  as when it is 250  $\mu\text{M}$ . Increasing potassium will increase potassium absorption, regardless of the addition of calcium. Hiatt (1969) showed that potassium and sodium are also competitive, probably for a common uptake site, in barley roots, with potassium being favored by some mechanism resembling a feedback sensor at lower concentrations. Thenabdu (1968), indicated that magnesium plays an important part in this process, even at uptake, in the cotton plant. Potassium was higher in the leaves of plants supplied with sodium, irrespective of magnesium; but sodium decreased the amount of potassium in the stems of plants supplied with adequate magnesium. A strong magnesium/sodium interaction influenced the movement of potassium to the meristems of the plant. Sodium also increased the amount of calcium in all tissues of plants with adequate magnesium, but had the opposite effect if magnesium was limiting. Rains (1969), working with bean stem slices, showed that potassium absorption increases in aging tissues, whereas sodium absorption decreases. The application of cytokinin suppressed the absorption of potassium but had no effect on sodium absorption.

### Antagonisms

Closely related to the kinds of relations between minerals found in uptake are phenomena that have been called "antagonisms." It has been shown that the property of divalent ions, particularly calcium and magnesium, to reduce the toxic effects of the monovalent ions, potassium and sodium, seems to have a functional basis apart from uptake phenomena. The hypothesis that the active site of this action is at the cell membrane, where divalent ions would serve to tighten membrane binding, and monovalent ions to weaken it (see Bonner and Galston, 1952), was not widely accepted because of the disproportionate amounts of divalent ions necessary to counteract the damage, i.e., amounts of divalent ions far smaller than their equivalents of monovalent ions, are effective. A recent result (Nieman and Willis, 1971), however, showing that "the correlation between the release of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  and release of protein, and between these effects and the suppression glucose and orthophosphate uptake, supports the hypothesis that divalent ions maintain, and monovalent cations disrupt, linkages between the outer cell surface and proteins required for active solute uptake."

### Mechanisms of internal control

It would not be unreasonable to suppose that the role of all ions in normal plant metabolism form a combined system of internal control to regulate the internal distribution of ions in view of plant needs. Distributions other than those we might deduce from this normal system could be attributed to plant adaptations to a unique environment. These mechanisms have primarily been studied with regard to selective ion transport from the soil to the plant in different environments as it leads to the formation of ecotypes for this character. Epstein and

Jeffries (1964) reviewed the genetic basis of selective ion transport in plants, with particular reference to acid-calcareous differences involving the calcium ion. Snaydon (1970) studied "mosaic differentiation" of ecotypes in a mosaic acidic-calcareous soil in 40 years over a distance of 30 meters. Other studies have utilized ion accumulation characteristics, particularly acid-calcareous adaptation and heavy metal tolerance, to model the mathematics of evolutionary differentiation in short periods over short distances (Antonovics, 1968; Jain and Bradshaw, 1966; McNeilly, 1968).

Kleese, Rasmusson, and Smith (1968), using 22 plant varieties over two years at two different locations showed that the primary factor affecting the accumulation of phosphorous, potassium, magnesium, sodium, calcium, manganese, boron, and strontium is genetic and not environmental. Very few gene-environment interactions were significant and genotypes were consistent over time and space. High correlations found between seed and seedling leaf accumulation between calcium and strontium, magnesium and calcium in barley, and magnesium and strontium in wheat, were thought to represent systems of linked genes.

Specific work on the nature of high, medium, and non-calcareous soil ecotypes has been done by Ramakrishnan and Singh (1966). They demonstrated that in edaphic ecotypes of Cynodon dactylon, ash content and uptake of calcium, potassium, and magnesium generally increase with increasing soil calcium; except with the medium and non-calcareous types in high calcium soil. These experiments showed that ecotypes differed markedly due to a soil calcium response. Further experiments (Ramakrishnan, 1968) have shown that the ecotypes also differ in response to pH and phosphorous availability.

Direct physiological mechanisms for this differentiation have been less widely examined. Jeffries et al. (1969), however, have shown that clones of Lemna minor, growing in different calcium regimes, differ in the response of the enzyme malic dehydrogenase to calcium ions. While this in itself seems to be primarily a phenotypic response, there is some indication that changes in enzyme configuration, and affinity for the ion, represent a basis for ecotypic differentiation.

Using these ideas, I have developed two approaches that may afford a more systematic look at the internal systems of ion control, the reactions on which these processes of differentiation will occur. These systems represent mechanisms uniting the physiological role of the ion to the determination of its internal distribution and uptake characteristics.

1. A set of equations based on Jeffries' et al., (1969) presentation of Michaelis-Menten kinetics to describe ion uptake, adapted to describe internal ion transport, and expanded to include the concept of bound and unbound ion fractions in plant tissue, with a dynamic equilibrium between the two fractions, determined by ion requirements for usage in any given internal region at a given time.
2. A circuit analogue and differential equation set to describe the same system as 1, based on the technique of analogue modelling of biological systems as described in Blesser (1969).
  - (a) Epstein and Hagen (1952) used an equation of the same form as the Michaelis-Menten equation for enzyme kinetics, to describe ion uptake at the plant root. Plotting the data according to this formulation, Epstein (1966)

elucidated the dual mechanism of ion absorption mentioned above. In the equation,

$$v = \frac{V_{\max} S}{K_m + S}$$

$v$  is the rate of uptake by the plant at external concentration  $S$  of the ion.  $V_{\max}$  is the maximum rate of uptake, and, according to the Michaelis-Menton theory,  $K_m$  is a measure of the affinity of the carrier protein for the ion.  $K_m$  then is the quantity which differed for the two absorption mechanisms Epstein found. Jeffries then applied this equation to distinguish two  $K_m$  values for potassium in two edaphic ecotypes of liverwort.

The ion pool within plant tissue may be roughly divided into two complements: bound, or osmotically inactive; and unbound, or osmotically active. In the root uptake process, this division has been characterized as "adsorption" and absorption." While the internal geography of these two complements has still not been clarified by research, we can approximately assume that ions that form loose bonds with larger molecules, e.g., hydration or easily reversible cofactor activity, will fall into the active category, whereas tightly bound ions, e.g., chelated, in hemes or poryphrins, will be inactive. The position of free ions in vacuoles (i.e., free in the cell sap) is not quite as clear. It may be that in some cases the cell exercises a mechanism whereby these ions may be compartmented and isolated without binding but by enclosure. No distinct dividing line exists between these

fractions, however, but rather a continuum of activities, proportional to diffusion barrier resistance. The two root fractions have been divided empirically by the discontinuity of efflux times (Fried, Noggle, and Hagen, 1958), and a similar process of separation should be possible for non-root tissues, perhaps by homogenization times, time or strength of washing with cold hydrochloric acid, etc. The rates at which an ion species is converted from one fraction to another would then be measurable, and this I would propose to be a measure of the usage or storage of the ion in metabolic pathways, in any given region of tissue.

We might similarly use Michaelis-Menton kinetics to describe this conversion, uptake into the unbound fraction, and acquire a  $K_m$  for that affinity, perhaps a measure of the need for that ion in the metabolism of a given organ, in a plant growing in a particular space, or at a particular time of the plant's development.

If (A) represents the unbound fraction, and (B) the bound fraction, then  $A + B$  will equal (C), the total concentration of the ion in that tissue. Hence,  $\frac{d(B - A)}{dt} = (u)$ , the rate of usage or storage at that site. The equations,

$$u = \frac{U_{\max} A}{K_m + A} \text{ for binding, and } u = \frac{U_{\max} B}{K_m + B} \text{ for release,}$$

will be equations for those terms analogous to the one described above. The same kinds of determinations should be possible. This more detailed examination of ion control mechanisms should provide a finer edge for examining those ecotypes that

differentiate according to ion responses. By stressing particular organs or ion requiring processes, we could examine phenotypic responses. Or, to combine the two, certain ecotypes may differ, for example, in the affinity of a particular organ for a particular ion at a particular time in development. There is some indication in the data for this thesis that such a mechanism may be involved in the endurance of low temperature stress by the plants studied.

- (b) The techniques of analogue modeling employed for part (b) make use of the fact that the dynamic properties of electrical elements may approximate those of certain broader physical processes. In this model we make use of the two most basic of those properties, resistance and storage. Resistive properties can be characterized as those analogous essentially to the process of friction. The power differential of what is designated as the "across variable" is proportional to the translation of that energy into some "through" variable." Resistance is the measure of the efficiency of that translation. In electrical processes the across variable is voltage and the through variable is current. In hydraulic processes these would be pressure drop and liquid flow, respectively. If we plot the across variable on the x axis and the through variable on the y axis for a linear process, resistance will equal the inverse of the slope of a line designating the system characteristics. In an analogue model an element of the system exhibiting this behavior would be modelled with an electrical resistor, and the variables described by properly scaled

quantities of current and voltage as described above would exhibit the behavior of the through and across variables of the system being modelled.

Storage, or compliance, represents the property of physical systems in storing mass or energy. In the hydraulic model, for example, rather than a rigid tube in which flow and pressure drop are inflexibly related by a resistance equation, storage properties would be analogous to flexible elements which could store liquid. It can be represented mathematically as the volume stored, which is the integral of the through variable in the resistance equation divided by the same across variable. This property can be represented electrically by capacitors, in which case capacitance will reflect the storage property, and the integral of current flow and voltage represent the physical inputs to the element.

For this model of ion partition in plants, I have fashioned a circuit which should combine these elements in such a way as they are analogous to individual elements and their network of connections in the ion transport system of plant tissues, and then derived from this circuit a series of differential equations (see appendix) based on the properties displayed by that circuit. The circuit is analyzed by means of Kirchhoff's Laws. Loop equations make use of the fact that the total voltage drop around an enclosed loop is zero, and nodal equations make use of the fact that the sum of all currents entering and leaving a circuit node is equal to zero.



The across variable in this model are the differences in ion concentration that are related to the through variable of ion flow by the properties of resistance, the resistance to diffusional flow in plant tissue, and the storage of ions in any particular part of the plant. Electrical resistors represent resistance in any given circuit leg, and electrical capacitors represent storage capacity in any given leg.

The circuit is analogous to the parallel system of a power transmission line. Voltage loss in succeeding parallel loops represents ionic processes in organ regions at succeeding distances from the root-soil interface. Concentration differentials at this interface, active uptake processes, and the driving force of transpiration, are the power supply in this system. The dynamic processes of current and voltage drop represent static equilibrium concentrations of ions in the tissue, with proper scaling, as the system elements of each organ-loop are intended to represent only those processes that effect the steady state concentration of the mineral in each region. Line resistance in the circuit represents resistance to ion diffusion and determines the maximum possible equilibrium concentration possible in each area due to its position relative to the root, ignoring its metabolic activity. Resistance across the lines represents ion losses to irreversible binding. Capacitance across the lines represents reversible binding, whereby ions are removed from osmotic activity but remain recoverable for periods of high activity in the immediately surrounding tissue, or for transport to other areas in the

plant. It should be noted that these are not the same two fractions as defined in (a). The final circuit (Figure 3) is simplified to these basic components: Active uptake and passive uptake at the root, diffusion resistance in the stem above and below a given leaf, and usage and storage in the stem apex, leaf, and root. Diffusion resistance in the leaf, and usage and storage in the stem are ignored.

The current source ( $Y_g$ ) represents the combined uptake by both active and passive processes. Line resistances (i.e.,  $R_{fr}$ ,  $R_{sb}$ , and  $R_{sa}$ ) represent the resistance to diffusional flow in the center of a homogeneous stream (ignoring the electrical effects) according to Fick's Law,

$$K_1 - K_2 = (1/D \ x/A)w \quad (\text{see Blessner, 1969, p. 64}).$$

( $K_1$ ) and ( $K_2$ ) are concentrations on opposite sides of the diffusion barrier, ( $w$ ) is solute flow, ( $D$ ) equals membrane diffusivity, ( $x$ ) equals the length of the conducting path, and ( $A$ ) represents its cross-sectional area.

Across resistances represent the rate of binding into osmotically inactive forms ("irreversible"), and capacitances represent the rate of "reversible" binding.

The differential equation derived from this circuit may be useful in much the same way as the equations from part (a). In this case we have the added ability of estimating the internal distribution of an ion which would result from various rates of usage and storage at the plant organs. Conversely, given a pattern of distribution for any ion in

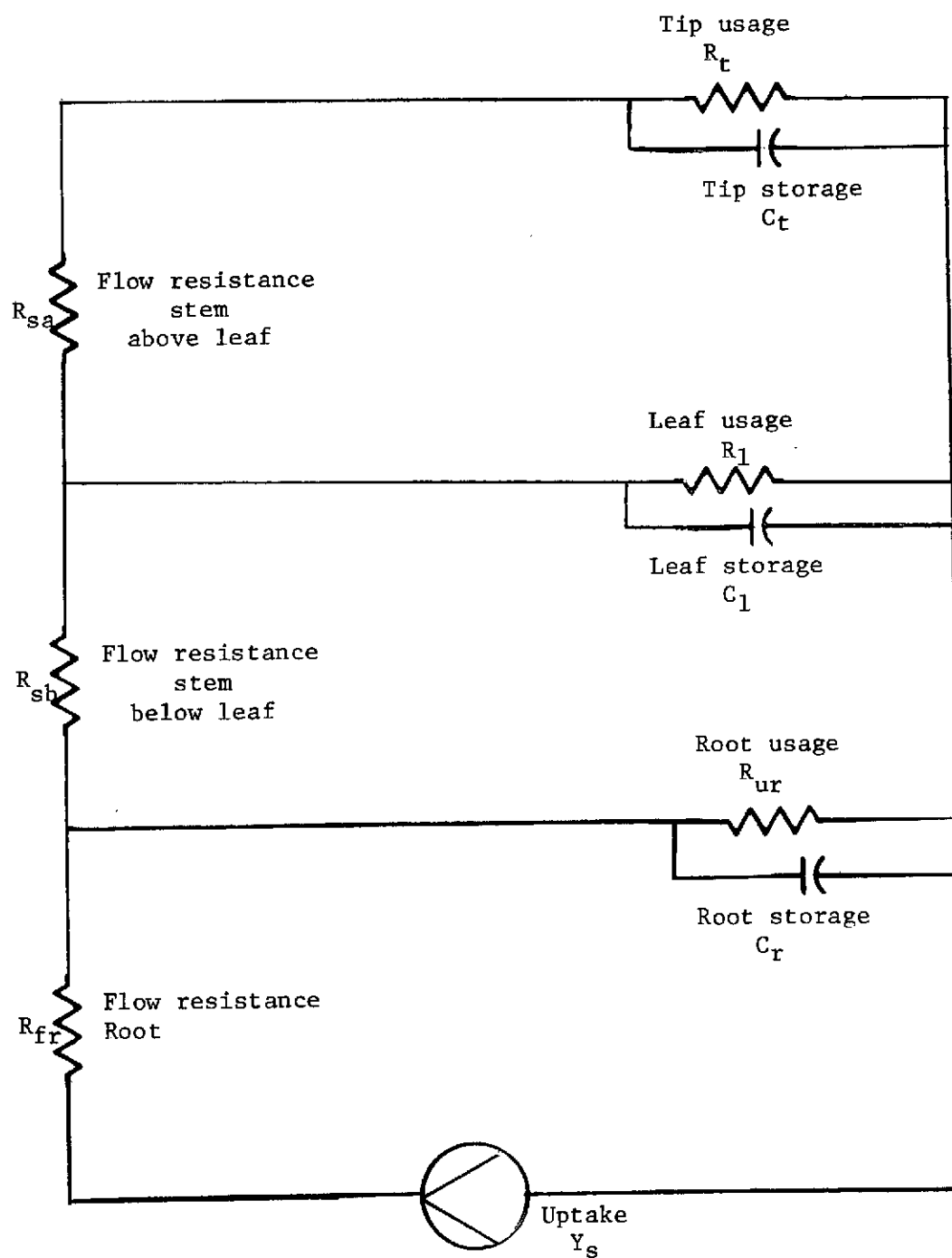


Figure 3. Analogue circuit for ion partition in plants.

the plant, we can deduce the rates of usage or storage in any organ. Differences in these rates may represent ecotypic adaptation in different environments.

#### Low temperature involvement

Such patterns of distribution were collected for this thesis. Because the primary stress factor for these plants is probably low temperature, and ions are involved at the membrane, the primary target of this stress, it is hoped that the adaptations of selective ion transport found represent mechanisms of low temperature endurance.

The specifics of ion distribution in protection from the cold have generally been investigated in terms of the biostructure theory mentioned above. I include in this not only the strengthening of protoplasm in Stocker's sense, but the osmotic effects of any solute in the cell sap, such as small ions, organic acids, free sugars, and soluble protein. Additional evidence indicates, however, that organic acids may act non-colligatively by interaction with specific sites on the membrane, and that inorganic salts may participate in this reaction (Santarius, 1971). Good (1962) has demonstrated that high anion concentrations in the medium will cause an uncoupling of the Hill reaction from photophosphorylation. Heber and Santarius (1964) demonstrated that cold temperatures will uncouple phosphorylation from electron transport in isolated chloroplasts and mitochondria. In non-cyclic phosphorylation no ATP synthesis is observed. In the light oxygen is evolved and electron transfer occurs. Uncoupling is complete in an isolated spinach chloroplast suspension in 2 hours at -25 C. The effect is independent of chloroplast concentration. Two percent sucrose will protect the

chloroplasts, but that protection is overcome by the addition of NaCl or  $MgCl_2$ . Izawa and Good (1966) have observed the effects of this uncoupling on the conformation of isolated chloroplasts in the electron microscope. In their study, chloroplasts isolated from low salt media exhibited the swelling and loss of granal structure which we have associated with a cold temperature or general stress response. Addition of NaCl or  $MgCl_2$  induced an intralamellar attraction that resulted in reorganization and stacking in either random or normal configurations. This effect is associated with electron transport but is apparently not important for the Hill reaction. The same membrane shrinkage does not occur if electron transport is induced with oxygen. Murakami and Packer (1971) attribute this structural change to an alteration of hydrophobic bonding in the thylakoid membranes, induced by the mineral cations.

Temperature will also affect the absorption of ions from the external medium, and thus plant viability. Korovin et al. (1968) studied the absorption of nitrogen, phosphorous, potassium, calcium, and sulfur, in 6-day-old corn seedlings. In general, absorption increased with increasing temperature. From 1 to 5 C, the absorption of most ions is unchanged, except for potassium which doubles in this interval. From 5 to 10 C, both calcium and potassium decrease. Around this point growth by division begins. The decreasing absorptions, particularly of phosphorous and calcium, may account for low temperature after-effects in treatments at this temperature. At 12 C absorption increases again, and for these treatments the after-effects disappear. Sutton (1969) indicates that although phosphorous absorption will decrease at low temperatures, as will the total amount of mineralized

phosphate available after conversion from organic forms, the lower rate of dry matter formation in the plant might actually allow for storage of surplus phosphate under these conditions in the field.

Mengel and Herwig (1969) studied the changes in potassium retention with temperature (in excised roots of young cereals) and its relation to respiration. Retention was higher at 2.5 to 10 C than it was at 15 to 30 C. Glucose and fructose addition increased retention and also respiration. Oxygen consumption, however, was not apparently related directly to potassium efflux. If respiration was enhanced with temperature, potassium retention decreased, but if enhanced with a glucose-fructose treatment, potassium retention increased. Retention capacity is also proportional to indiffusible anions. At higher temperatures their breakdown would lower potassium retention. With an electric potential gradient lowered by organic anion consumption, efflux will increase.

There is some possibility that sodium may play a significant role in low temperature response. Howell and Jung (1965) tried to correlate cold resistance in orchard grass with levels of potassium, calcium, sodium, and nitrogen. Though there is some difficulty in interpreting their results (inasmuch as sample date is an unaccountable source of variation) sodium did change drastically during growth. Kuiper (1967) found that monogalactose diglyceride will also affect sodium transport, not generally, but at least in cotton. Sodium then might be related to lipid metabolism for membrane repair in cold stress.

The ion that is most effective in the antagonistic effects of divalent cations (see Bonner and Galston, 1952), and that is most suited to be a protective agent from cold temperature in membrane

manifestations, is calcium. There is some indication from purely distributional ecology (Fernald, 1919) that calcium in limestone soils can compensate for cold temperatures to widen plant ranges. Chang, Lowe, and Hiatt (1968) have studied calcium distributions in plants subject to high temperature stress. High potassium in these conditions can be a contributing factor to calcium deficiency. Increasing calcium deficiency is noted with increasing temperatures from 21 to 30 C. Decreasing calcium in stems and leaves is associated, under these conditions, with increasing potassium in these regions. Meristems and terminal leaves are the critical areas for calcium deficiency and can sometimes be adversely affected by accumulation of calcium in the stem. Epstein has compared ratios of three ions (potassium, calcium, and magnesium) in tops and roots of the potato plant, as they vary with temperature. The top to root ratio of ion concentration increases for all three elements with a temperature increase from 5 to 29 C. Potassium and magnesium concentrations increase in the top while decreasing in the root. Calcium concentrations increase in both root and shoot.

The regional antagonisms, as noted by Chang, Lowe, and Hiatt (1968) during high temperature stress, are quite reminiscent of the functional antagonism of potassium and calcium that Nieman and Willis placed at the membrane. Jones and Lunt (1967) in reviewing the function of calcium in plants, pointed to the indications of its primary role in protecting membranes, citing for example the work of Bushueva and Semikhatova (1965) demonstrating cristae swelling in calcium deficient plants. Epstein (1961) described the effectiveness of calcium in maintaining the integrity of the selective

absorption mechanisms for potassium and sodium (at concentrations of  $1 \times 10^{-3}$  M). In these studies manganese had limited activity, and magnesium was ineffective.

These parallel results emphasizing the specificity of monovalent-divalent interactions at the membrane and thus as a crucial factor in membrane reactions at low temperature, are supported by a multitude of studies touching on all aspects of membrane phenomena in plant cells. Gomperts, Lantelme, and Stock (1970) have established the physical basis for these interactions in extracted by structurally intact membrane fragments. Using the fluorescent probe, 8-anilino naphthalene-sulfonate (ANS), to measure the number of apolar binding sites on the membrane, they suggest that divalent cations bind to membranes at four distinct sites with varying association constants. The characteristics of titration suggest that only one species of binding site is available at any one time, and open the possibility that structural transitions of the unassociated coordination sites may be induced by divalent cation binding. Monovalent cations bind endothermically, and divalent cations have both endothermic and exothermic binding reactions at different sites. Monovalent ions affect divalent cation binding by reducing the activity coefficient: they do not appear to displace divalent cations from their binding sites. This seems to be a qualitatively different phenomena than the type of divalent binding that occurs in synthetic lipo-protein films, in vitro, to increase structural integrity (Gurd, 1960). but this too would represent a direct physical basis for divalent cation action.

Calcium and potassium seem to be the most potently antagonistic of their respective species, in my work, and in some I have cited above.



The specificity of the interactions between these elements is discussed by Resnick, Lunt, and Wallace (1969). In these studies, transport and uptake, and generally all the temperature dependent processes involving calcium and potassium, hold equally well if substitutions are made for either of the two ions with their structural analogues, cesium and strontium, respectively. Uptake inhibition reactions do not hold, however, which raises the possibility that these inhibitions too are not "non-functional" antagonisms in the medium, but rather a special case of the membrane interaction, perhaps at the root membrane at which uptake occurs.

Work by Sipos and Meckel (1968) points up this specificity in calcium activity and reminds us of the kinds of ecological considerations discussed above in the work of Snaydon, Jeffries, and others on edaphic ecotypes. This work (Sipos and Meckel), done on animal enzymes, shows that with changing temperatures the enzymes trypsin and bacterial proteinase undergo unexpected structural changes. With high calcium present a new conformation of the enzyme is stabilized, and new activity occurs. This is shown to be more than just a protection from denaturation, and a host of other divalent cations, e.g., magnesium and copper, are ineffective.

It would appear, then, that in their effects on the growth of plants at low temperatures, magnesium and sodium will be most important in their uptake and transport interrelationships with other ions. Sodium may possibly be important in regulation of lipid transport and thereby membrane repair. Potassium will be important through its antagonism to the calcium ion at the membrane and organ levels.

Calcium may be involved in primary endurance mechanisms through its structural properties in combining with protein molecules in either enzymes or membranes.

## MATERIALS AND METHODS

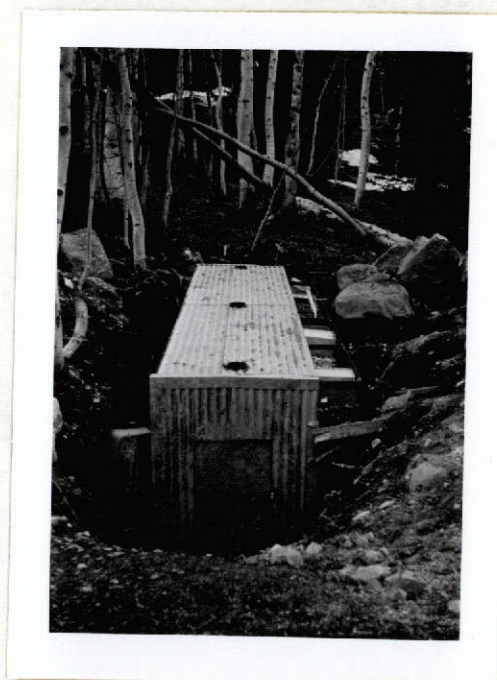
### Growth under Snow

#### Field studies

The site chosen for field studies of plant growth under snow was an aspen grove in Steep Hollow, Cache National Forest, known to support a large natural population of Claytonia lanceolata. Experimental facilities were deployed on a hillside of 25 percent slope with a northeast exposure at an elevation of 7700 feet. A tunnel, 6.1 meters long, 1.8 meters high, and 1.2 meters wide, was constructed of steel and buried parallel to the ground surface. At the top of one side of the tunnel runs a steel screen balcony with 2.5 cm of coarse gravel in the bottom and 20, 0.3 cubic meter, steel trays sitting in it with their tops at ground level. The trays were accessible in winter from underneath the snow through doors on the inside of the tunnel. The gravel could be scooped out so that the trays would drop to facilitate removal and replacement without injury to the plants. Half of the trays were provided with lids as dark controls. Lids were constructed to allow gas exchange. Snow depth was measured with 3, 2.6 meter, stakes marked at 0.3 meter intervals and attached to the tunnel as seen in Figure 5. Three portholes in the top of the tunnel allowed studies of light penetration of the snow.

In September of 1970, half of the trays were planted with bulbs of Galanthus nivalis obtained commercially, and half with corms of Claytonia lanceolata dug in the immediate area of the tunnel. Claytonia corms were planted 7.6 to 12.7 cm deep, approximately 50 per tray.

Galanthus bulbs were planted 3.8 cm deep, 17 bulbs per tray. Plants from these trays were used for a variety of experiments.



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Figure 4. Tunnel, uncovered.

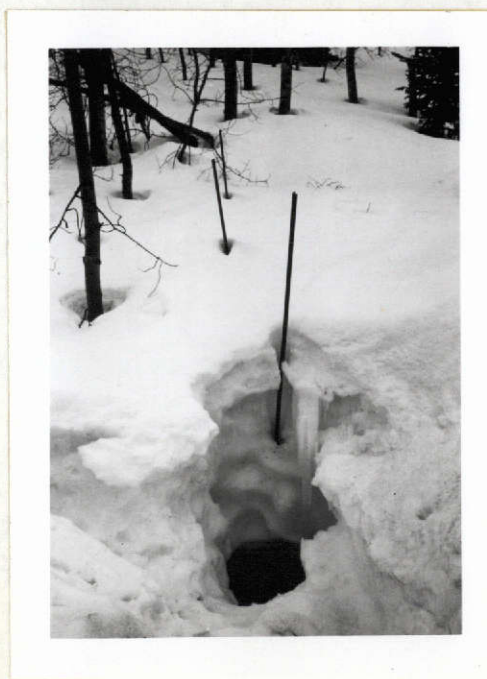


Figure 5. Tunnel, covered.

### Chlorophyll studies

Chlorophyll determinations with this tissue were done in total darkness throughout. Ten millimeters of an extraction solvent consisting of eight parts acetone to two parts 0.05 M  $\text{NH}_4\text{OH}$  was ground by hand with 1 gm. of tissue in a mortar and pestle for two minutes. This extract was centrifuged at 30,000 g for ten minutes, washed once in the extraction medium, and resuspended in the same solution. Samples were assayed for chlorophyll (a+b) and protochlorophyll in a Beckman DB-G grating spectrophotometer (see Figure 6 for spectra obtained under these conditions) according to the following equations:

$$\text{Chlorophyll (a+b) mg/l} = 8.44 (A_{663}) + 20.95 (A_{645}) - .62 (A_{626})$$

$$\text{Protochlorophyll mg/l} = -3.99 (A_{663}) - 6.76 (A_{645}) + 29.6 (A_{626}).$$

All absorbances (A) are corrected by subtracting absorbance at 700 nm.

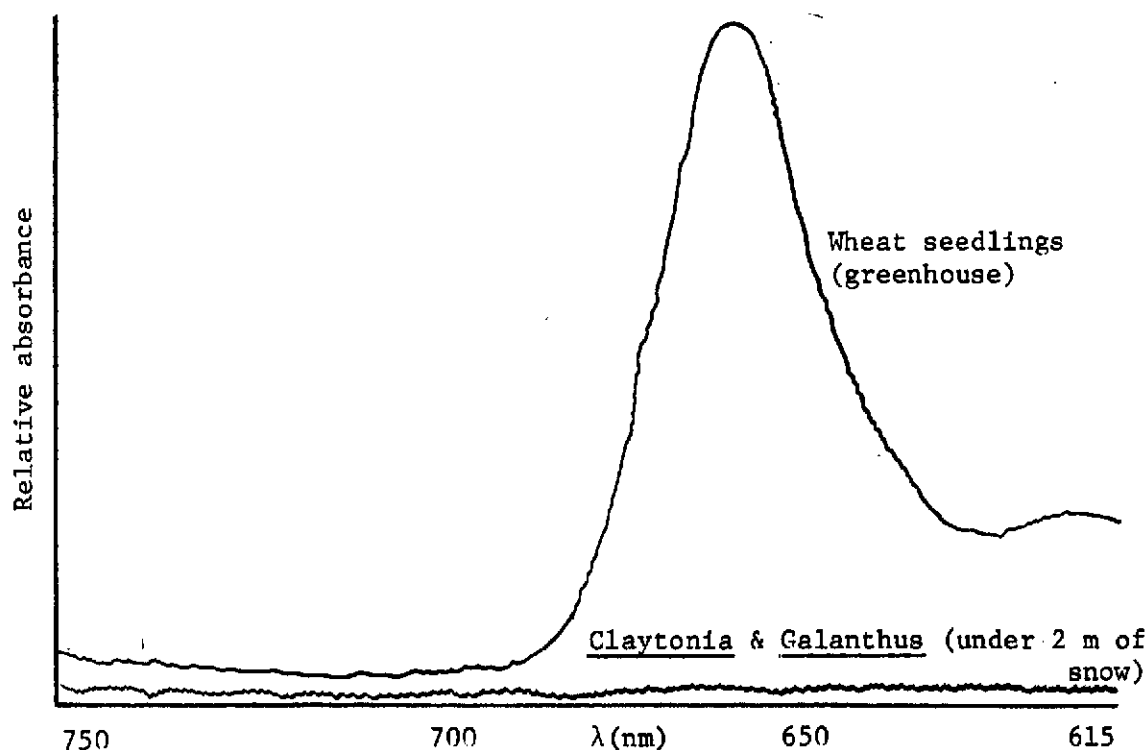


Figure 6. Extracted chlorophyll.

## Stress Protection

### Cold temperature protection of

#### Axonopus affinis by cytokinin

Experiments on the protective effects of cytokinin from cold were done with 2-week-old seedlings of Axonopus affinis (carpet grass). The seed was germinated in 4 inch square pots in the greenhouse in flats of 12 pots per flat. Cytokinin treated plants received  $2 \times 10^{-5}$  M Kinetin (6-Furfurylaminopurine) in all water supplied by subirrigation during germination. No foliar spray was applied to these plants. Two-week-old seedlings were exposed to treatments of 3, 9, 18, and 24 hours of -5 C, in a growth chamber with 65 foot-candles of fluorescent light, and then returned to the greenhouse for 24 hours. After that time plants were transferred into dark chambers at 1.5 C. Mortality was determined by visual examination. The distinction was fairly easy because most seedlings were either completely brown and withered or completely green and viable after 24 hours of greenhouse exposure. The few indeterminate seedlings in each treatment were discarded, there being no preponderance of such seedlings in any treatments. All data represent an average of 12 pots, each containing approximately 100 plants. Pot positions, after germination, were randomized in regard to the trays for all cold treatments.

#### Cytokinin and lipids in Spinacia

##### oleracea

Spinacia oleracea L. was planted in the greenhouse on May twenty-fifth in 4-inch square plastic pots, 17 plants per pot, in groups of 12 pots per tray. During germination all plants received

17 hours of light per day (including artificial illumination during the night). Plants were treated with cytokinin by the method of Kuraishi et al. (1966) receiving a foliar spray of  $2 \times 10^{-5}$  M cytokinin. Kuraishi used Benzyladenine, but in this case we used 6-Furfurylamino-purine. Beginning on June 15, 1971, treated plants received this spray, until wet, every 4 days until cold treatment, and controls were sprayed similarly with water. On June 29, 1971, all plants received 3 hours of -2 C. The fresh weights of sample plants were determined before cold treatment and 7 days after cold treatment. At this later time, tissue samples were taken from the third leaf of each plant at mid-leaf adjacent to the mid-vein. These samples were observed by electron microscopy for membrane integrity, starch storage in the chloroplasts, and lipid storage in the chloroplasts. Electron microscopy was done by Dr. Steven Kimball according to techniques described in his doctoral thesis (Kimball, 1971).

### Minerals

#### Antagonisms

Experiments on antagonistic phenomena were done with 2-week-old seedlings of Phaseolus vulgaris. Beans were germinated in sand until 2 weeks old, separated from the sand with care to <sup>maintain</sup> ~~avoid~~ root systems intact, and weighed after roots were rinsed with distilled water. Seedlings were randomized into treatments, 12 plants per treatment, and placed in 30 ml test tubes in the greenhouse. Each tube was filled with a solution of the chloride salt of the applied cations at the concentrations indicated on the graphs. Tubes were maintained to a liquid level above the seedling root with distilled water, thereby

lowering the concentration in the medium by the amount transported into the plant. No covering was placed on the tubes to prevent heating of the water by light transmitted through the glass tube. Data represent fresh weight changes after 1 week of this treatment in an average of 12 samples.

### Field studies

"Mountain" plants were removed periodically from the tunnel facility and brought down to the laboratory intact in their trays in insulated containers. "Valley" plants were collected on the bench above Richmond, Utah. "Immature" plants were collected from the edges of receding snowbanks. Their flower buds were formed but unopened and they were characteristically a dominant reddish color. "Mature" plants were collected after total snow melt, and were in full flower, though unopened buds remained on many plants.

Overwintering corms were assayed with growing shoot and roots attached (Table 4, see p. 57) or divided into tip, leaf, stem, and corm-root segments (Tables 1 and 2). Potassium was assayed by digesting 0.35 g of tissue in a mixture of 17 ml concentrated nitric acid and 3 ml of 70 percent perchloric acid. Volume was reduced to 2 ml, 10 ml of water added and the solution boiled and filtered through Whatman No. 42 filter paper. Sodium was treated similarly, except that the addition of 10 ml water was eliminated. Tissue digestion for divalent ion determination was done in 4 ml of 7:1 v/v perchloric/sulfuric acid mixture, plus 15 ml of concentrated nitric acid, boiled and filtered. Calcium standards and samples were assayed<sup>in 5% LANTHANUM. All solutions were Assayed</sup> by atomic absorption spectrophotometry and all data are averages of 16 readings on each of two sample solutions.



Table 1. Monovalent ion distribution

	Percent $K^+$			Percent $Na^+$		
	Mountain		Valley	Mountain		Valley
	Immature	Mature	Mature	Immature	Mature	Mature
Tip	15	30	17	14	38	8
Leaf	46	45	39	76	53	73
Stem	5	12.5	22	2	1	9
Corm	34	12.5	22	8	8	10

Table 2. Divalent ion distribution

	Percent $Ca^{2+}$			Percent $Mg^{2+}$		
	Mountain		Valley	Mountain		Valley
	Immature	Mature	Mature	Immature	Mature	Mature
Tip	15	35	11	23	23	5
Leaf	6	4	6	13	35	25
Stem	34	46	48	48	28	32
Corm	45	15	35	16	14	38

Cold temperature protection of  
*Axonopus affinis* by calcium

Experimental methods for carpet grass protection by calcium are the same as those for cytokinin. Treatment consisted of the addition of 25 grams of calcium chloride per tray, instead of cytokinin, applied to the soil solution during germination. Calcium addition was made only once at the beginning of germination. Similar experiments were also conducted in which both calcium and cytokinin were applied by their respective methods.

## RESULTS AND DISCUSSION

### Growth under Snow

#### Field studies

The snow depth curve in Figure 7 at the tunnel site in Steep Hollow demonstrates the objection to through-snow-light effects on cold resistance which is based on the time period involved. Although snow cover was abnormally high during the 1970-71 season (about 166 percent of normal), the general parabolic shape with shoulders during buildup and a steep melt gradient is probably typical. It is this steep melt gradient which means that the time during which snow depth is low enough to allow any of the through-snow-light effects to occur is very short. Light visible to the human eye has been reported in the past through 1.9 meters of snow, by crawling under the snow pack and covering the entrance hole with black plastic (Kimball, 1969). Without this three-dimensional diffusion surface, however, and perhaps because of the uniquely icy composition of the snow during the 1970-71 season, no light was visible at depths greater than 0.9 meters through port-holes in the top of the tunnel. As indicated on the graph, this depth occurred approximately 2 weeks prior to total melt. Using an ISCO Model SR spectroradiometer, no light was detected penetrating the snow pack before this time. While two weeks may be important to plant development in some cases, it is questionable, given the observations of Billings and Mooney (1968) mentioned above, that it would constitute an important time period in this situation.

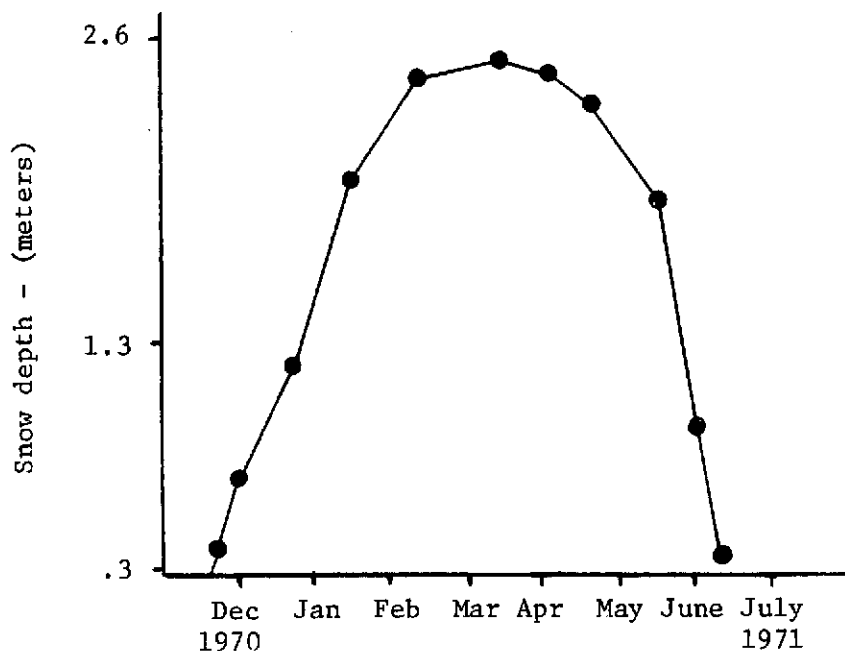


Figure 7. Snow depth. Variation with time.

#### Chlorophyll studies

Tissues of both Galanthus and Claytonia were assayed for chlorophyll during the period 3/4 to 4/17, when snow depth varied from 2.1 to 2.3 meters. Two samples of each species were taken on three occasions during this time. All treatments showed a total lack of protochlorophyll, chlorophyll a, and chlorophyll b (see Figure 6). It is not surprising that no chlorophyll is present at this time, since no light can penetrate snow of this depth, and we have no reason to believe that these plants are capable of converting protochlorophyll to chlorophyll in the dark. No visible greening had occurred by the time the light had reached the 0.9 meter level (June 28), indicating that no synthetic process had begun by this time. Dark grown controls observed at total melt (July 13) showed marked etiolation, but total growth was equal to

light controls. Fresh weight was greater in dark controls, due to longer stems, but differentiation of floral parts was less advanced. This confirms earlier studies (Kimball, 1969; Bennett, 1971) indicating that light reception and chlorophyll synthesis by these plants is a part of their early morphogenetic development. The fact that etiolation syndromes under these conditions do not differ appreciably from those which are found in normal environments, however, suggests that this light reception is not linked to any specific mechanism of cold temperature resistance or endurance. Since even normally etiolated plants would not survive the entire season, there is no way we can entirely rule out a resistance effect. Endurance has been defined, however, as the ability to grow at freezing temperatures. In these plants, that has been interpreted to mean specifically differentiation at the meristem (Kimball, 1969). Since dark grown plants continue to demonstrate endurance properties, we can rule out involvement of light in these endurance mechanisms. Light may modify their action, of course, but it is apparently not crucial to their existence.

In an attempt to examine resistance properties further, covers of some dark controls were removed on June 28 with snow at 0.9 meters. At total melt these plants were not visibly different from plants uncovered all winter. This confirms the lack of any through-snow light reception before this time. Though lack of material prohibited the exercise this year, further experiments may be feasible that could expose these plants at later dates in hopes of delineating some cutoff point, in time, for through-snow light effects.

## Stress Protection

### Cold temperature protection of

#### *Axonopus affinis* by cytokinin

The study of possible endurance mechanisms involves the hormone cytokinin because of its implication in the syndrome of low temperature damage as discussed above. Although it is inconvenient to conduct laboratory studies on either Claytonia or Galanthus, because of their necessary year-long growth cycle, it is hoped that growth of rapidly dividing young grass seedlings will approximate events in the meristematic tissues of those plants.

The left portion of the graph in Figure 8 shows the mortality response, after 24 hours in the greenhouse, of seedlings exposed to varying amounts of extreme cold stress. Part of the response may be due to the transfer from low intensity light to high intensity light. While the light effect may be an additional stress factor, we are evidently in the steep region of the stress response curve, where the extreme slope indicates a linear response. If there is a lag in the response to stress, cytokinin does not seem to have any qualitative effect on it. It evidently does, however, protect the plant from stress, lowering the slope and the final mortality without changing the course of the response in any obvious way that might give us a clue as to its mode of action.

The right portion of the curve shows the mortality of plants transferred into the dark at 1.5 C for varying amounts of time after the above treatment of 24 hours extreme cold. The control plants continue to suffer from increasing cold damage. Apparently there is no hardening response under these conditions. In this portion of the

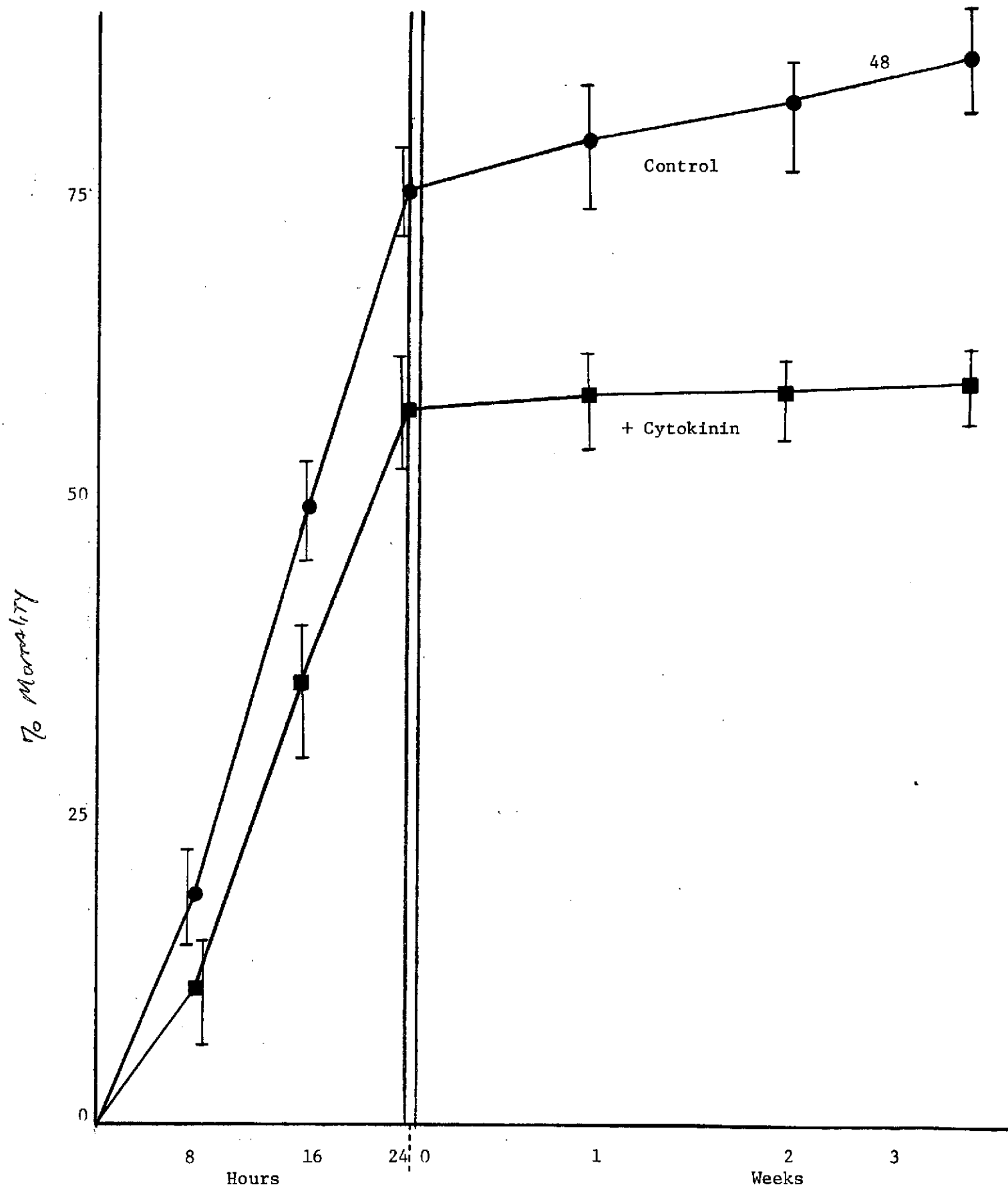


Figure 8. Cold temperature protection of *Axonopus affinis* by cytokinin.

curve, however, cytokinin treated plants are less susceptible. These plants, which are no longer receiving cytokinin, though both treatments receive water, show a continued protection from cold, without the increase in mortality seen in the controls. If the quantity of cytokinin applied is not in excess for the noted effect, and it is lost from the system by degradation, this might suggest that cytokinin is a trigger for some protective process in which its continued presence is unnecessary. If protection, for example, is proportional to some protein substance produced in the plant, we might conclude that the increased amount of this substance produced in the light, in the presence of cytokinin, is stored in the plant for later use, or that it can continue to be produced in the dark by plants pre-treated with cytokinin, whereas it cannot be by untreated plants.

#### Cytokinin and lipids in *Spinacia* *oleracea*

To examine the mechanism of cytokinin action in low temperature protection, I used a variation of the original experiments (Kuraishi et al., 1966) which demonstrated this protection. Pictures published by Kuraishi show healthy plants of *Pisum sativum* 7 days after cold treatment, for the sample treated with cytokinin, and severely wilted plants for those left untreated. Our experiment showed no such protection, probably due to the additional stress of the long-day exposure during germination of spinach, a long-day plant. This disruption of normal flowering cycles yielded plants that developed flowering spikes during early growth, far too early in the growth cycle for much leaf production. Kuraishi reported on this procedure in a variety of plants, germinated under both winter and summer light regimes, and for certain



species he too noted this interaction. Spinach, however, was one plant that he reported as exhibiting the same response in both regimes. Thus, it appears that these extremely long days represented an even more severe stress than his plants experienced.

Electron micrographs of our plants revealed healthy cell membranes and abundant starch storage in both treatments. The mean number of lipid globules in the chloroplast for each treatment was significantly different at the 5 percent level, as shown in Table 3. The greater number of lipids in cytokinin treated plants agrees with the two theories mentioned above that attribute increased lipid synthesis to cytokinin. It appears in this case, then, that this aspect of cytokinin action alone cannot result in stress protection, though this may result, in this case, from the unusual and extreme nature of the stress situation.

Table 3. Lipid globules per chloroplast

Cytokinin	
	<div style="display: flex; justify-content: space-around;"><span>+</span><span>-</span></div>
$\bar{X}$	<div style="display: flex; justify-content: space-around;"><div style="border: 1px solid black; padding: 5px;">9.3</div><div style="border: 1px solid black; padding: 5px;">6.2</div></div>
n =	<div style="display: flex; justify-content: space-around;"><span>18</span><span>18</span></div>
Ex =	<div style="display: flex; justify-content: space-around;"><span>112</span><span>168</span></div>
Ex <sup>2</sup> =	<div style="display: flex; justify-content: space-around;"><span>804</span><span>1692</span></div>
s <sup>2</sup> =	<div style="display: flex; justify-content: space-around;"><span>6.29</span><span>7.29</span></div>
S <sub>d</sub> = .85	
K = 3.63	
p(H <sub>0</sub> ) = .0011	

## Minerals

### Antagonisms

Mechanisms of low temperature endurance which involve mineral ions should involve the activities of these ions at the membrane. The experiments presented in Figures 9 to 12 are designed to examine the internal, or functional, aspects of monovalent-divalent ion antagonisms. The generally toxic conditions should limit the extent to which the results reflect active uptake interactions in the medium and reflect more closely the ion actions at the membrane. Care must be taken, however, to note that fresh weight will normally tend to decrease in all treatments of total molarity greater than 0.3 due to water loss from the tissue to the hypotonic medium.

In Figure 9, we see that the potassium-calcium antagonism is the most "effective" of the ones we are examining. While it does not give the greatest values for fresh weight increase, of the four cases, only in this one do we see an equivalence in the antagonistic effects, i.e., 0.3 M calcium must effectively counteracts the effects of 0.3 M potassium, and 0.6 M calcium most effectively antagonizes 0.6 M potassium.

The other three curves (Figures 10-12) show three qualitatively different kinds of pictures, none of which demonstrate the kind of equivalence seen in Figure 9. Magnesium at 0.6 M is a better antagonist for potassium than 0.3 M magnesium at all potassium concentrations (Figure 10), indicating perhaps that magnesium might act in the same way as calcium, but that more magnesium ions are necessary to perform the same function. The same kind of situation seems to exist in the

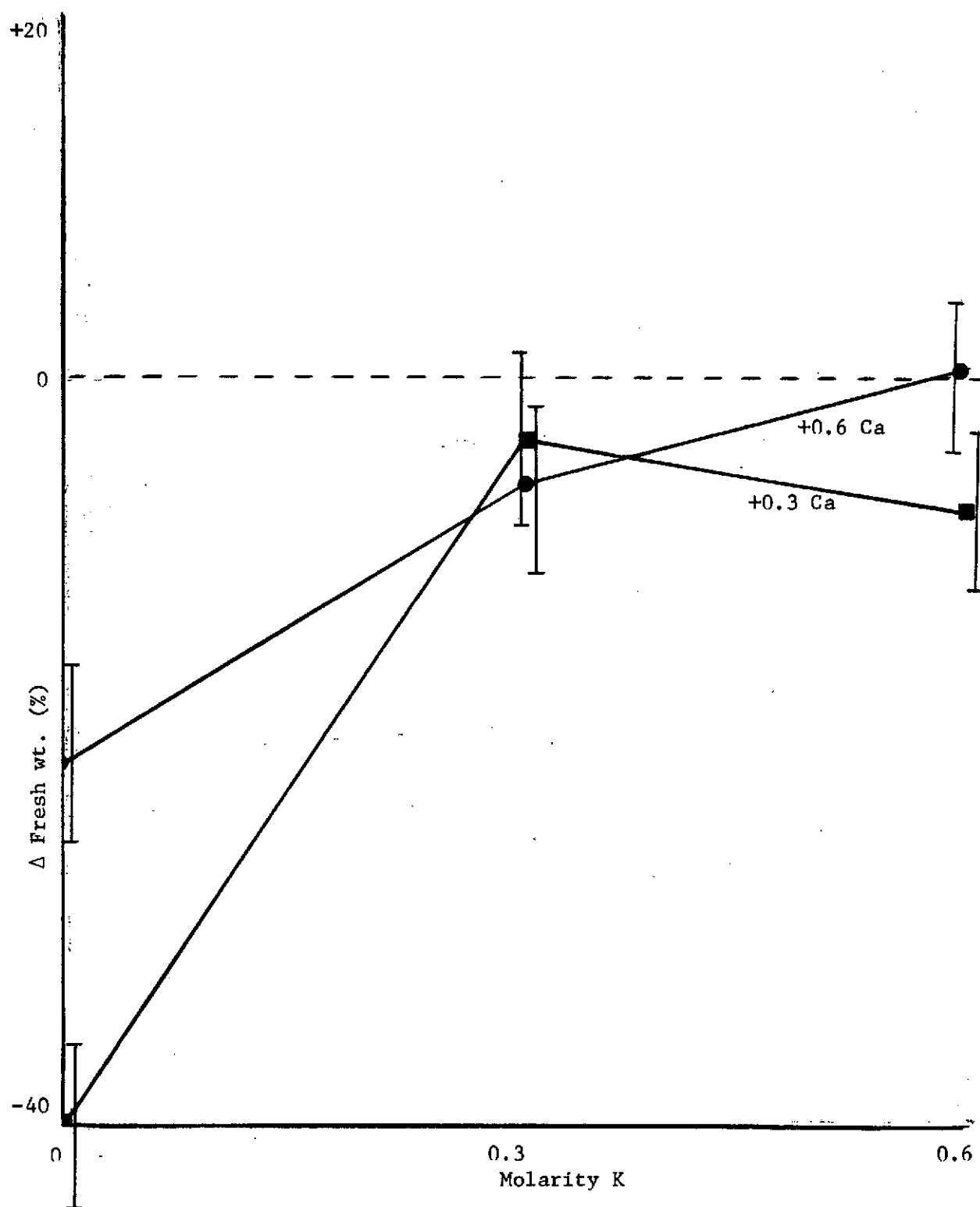


Figure 9. Antagonism: Potassium and calcium.

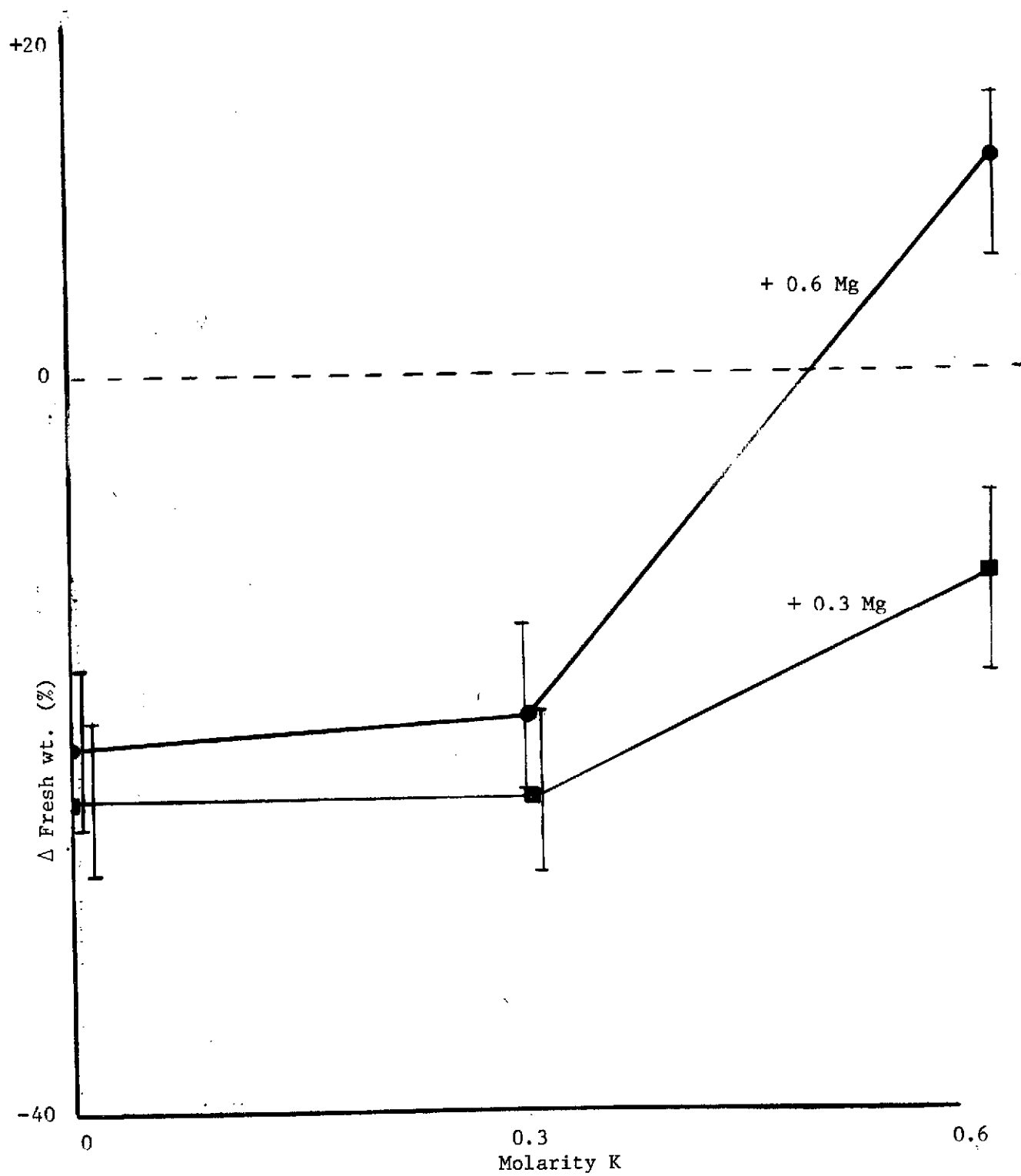


Figure 10. Antagonism: Potassium and magnesium.

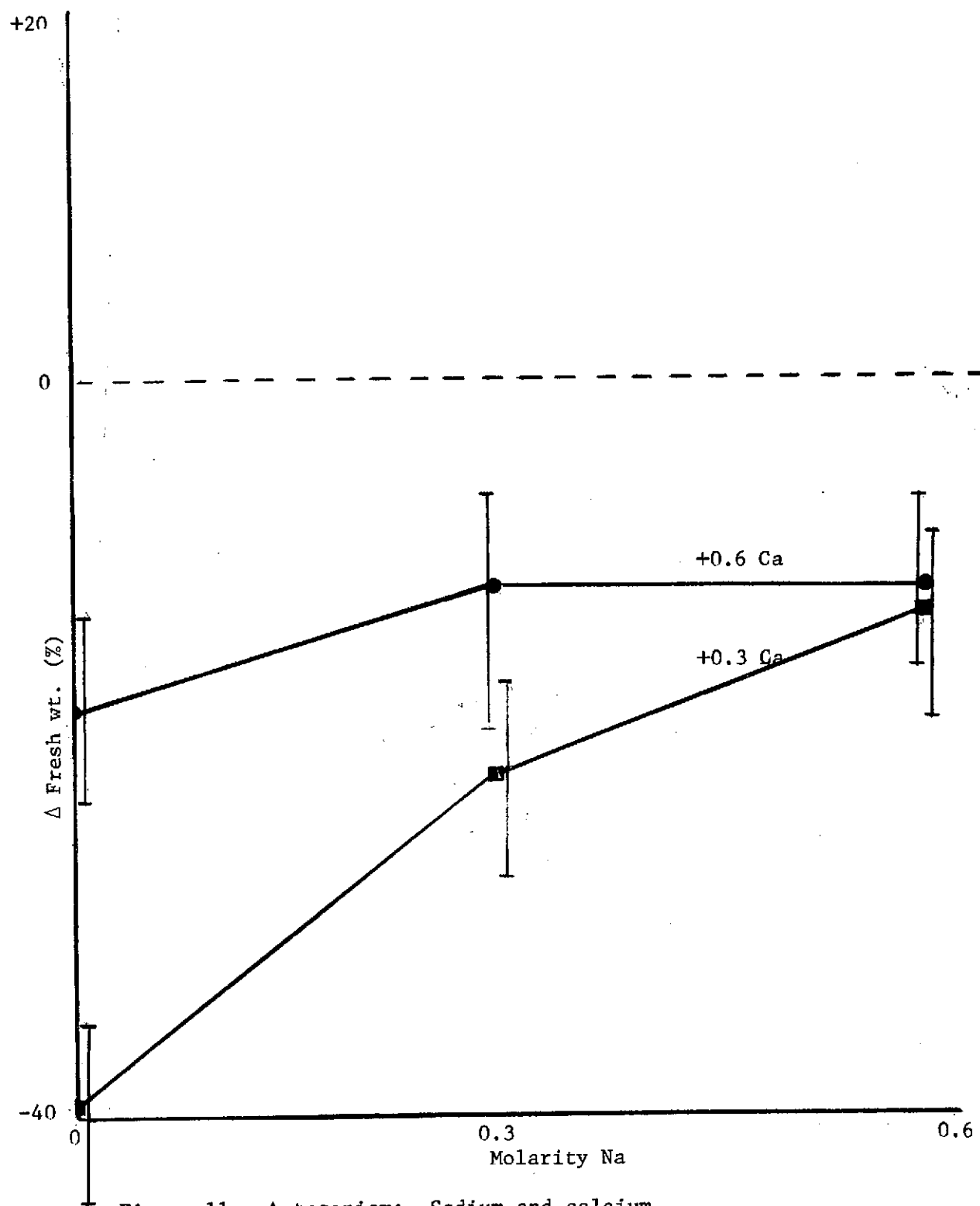


Figure 11. Antagonism: Sodium and calcium.

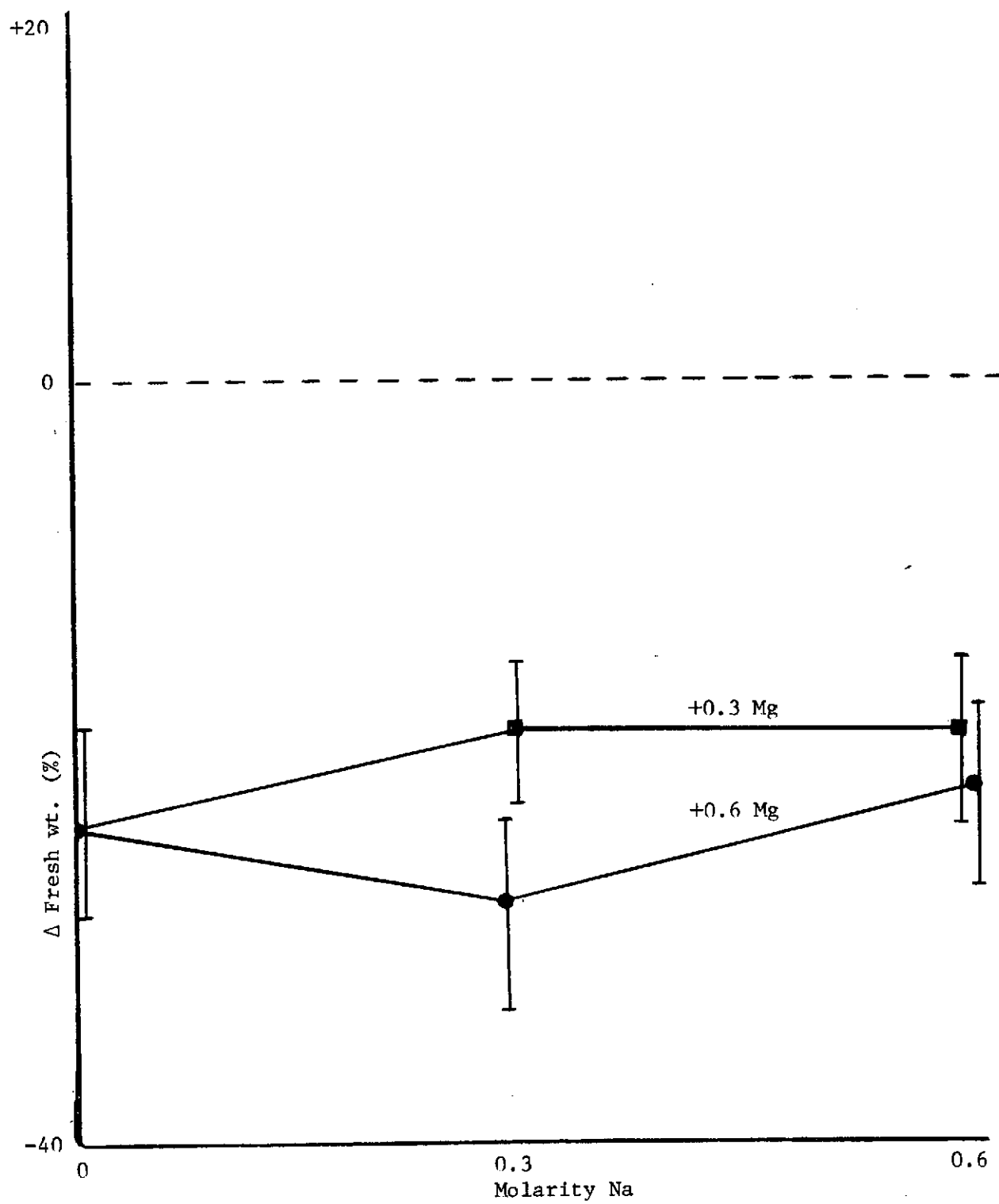


Figure 12. Antagonism: Sodium and magnesium.

antagonism of sodium and calcium (Figure 11), except that there is no increase in protection from 0.3 M to 0.6 M sodium if calcium concentration equals 0.6 M, while there is if calcium molarity equals 0.3. This would seem to suggest that there is a limited antagonistic activity in the interaction of these two ions, but there is no mechanism whereby it can pass this limited effectiveness toward equivalency. Figure 12 is more inconclusive. It could indicate that there is a certain antagonistic effect, if the two curves for 0.3 and 0.6 M magnesium cross at some point beyond the range of the graph, but it could also indicate that there is no antagonistic effect whatever, inasmuch as fresh weight changes for each sodium concentration are directly related to total molarity of the medium.

In view of the importance of the membrane site in low temperature endurance, these results emphasize that any antagonistic activities at this site in cold stressed plants would be most likely to involve the potassium and calcium ions.

#### Field studies

The field studies of low temperature stressed plants support this conclusion, and suggest that potassium/calcium antagonisms are connected to low temperature endurance.

Table 4 shows the general trend during maturation in *Claytonia* growing in the mountain environment toward a decreasing percentage of potassium and increase in other ions. This confirms other work noted above for a trend common to many plants. Comparison with valley plants may reflect differences in the life cycle timing (with earlier snow melt in the valley) or in soil composition at these sites. Differences

Table 4. Equivalent percent: Minerals in overwintering storage tissue

	Corm 11/21 - 6/13		<u>Claytonia</u> (whole plant)		
	<u>Claytonia</u>	<u>Galanthus</u>	Mountain		Valley
			Immature	Mature	Mature
K <sup>+</sup>	86	84	99.66	96.8	84
Na <sup>+</sup>	1.5	3.5	.05	1.4	1
Ca <sup>2+</sup>	9	3	.22	1.6	9
Mg <sup>2+</sup>	3.5	9.5	.06	0.2	6



in mineral composition of overwintering storage tissue between species suggest different mechanisms of ion uptake and storage involving calcium and magnesium, although potassium percentages are similar. This may be related to the fact that Claytonia plants used are naturally adapted to this high limestone, high calcium, environment, while the *Galanthus* is bred in Holland.

Tables 1 and 2 involve only the Claytonia plants. Here we can see patterns similar to the antagonistic processes noted by Chang, Lowe, and Hiatt (1968), whereby monovalent ions tend to accumulate in the leaves of stressed plants, and divalent ions in the tip. Potassium shows gross movement out of the corm into the tip as the plant matures, but stays higher in the leaf for both mountain and valley plants. Sodium exhibits similar behavior, though in the mountain plants more of it seems to move from the leaf to the tip during maturation. The total amount remains higher in the leaf, however, to a larger final percentage than potassium. Accumulation in the tip surpasses accumulation in the leaves only for the divalent ions, magnesium in immature plants, and calcium with an increasing trend toward maturity. This pattern appears to be a monovalent/divalent ion antagonism on the whole plant level, involving particularly the calcium and potassium ions.

This antagonism is one membrane effect which we might relate to low temperature endurance mechanisms. Closely associated with this phenomena is the selective movement of divalent ions, particularly calcium, to the tip. When analyzed according to the analogue circuit discussed in part (b) of the section on internal control mechanisms, this distribution indicates abnormally high elements of usage and/or storage for calcium at the tip. According to the equations in

part (a) of that section this indicates an active mechanism of calcium transport across some osmotic barrier, probably a membrane, since it is this meristematic region where we have identified the primary endurance abilities of these plants, any possible protection here would be strongly implicated in endurance mechanisms.

Cold temperature protection of  
*Axonopus affinis* by calcium

Experiments described in Figure 13 indicate that the calcium ion exhibits the same kind of protective ability as cytokinin (Figure 8). Although the general level of protection is higher for calcium, this may be attributable to the fact that the method of application is more suitable for calcium.

The experiment where both calcium and cytokinin are applied shows a protective effect averaging the two applied independently. This lack of synergism, or even additivity, does little to clarify the mechanism of protection for either agent. It does suggest that they are opposing in some way. It is reasonable to suppose that the mechanism of protection by calcium is related to its structural role in the membrane, particularly at transport sites, and in chloroplasts and mitochondria, as discussed above.

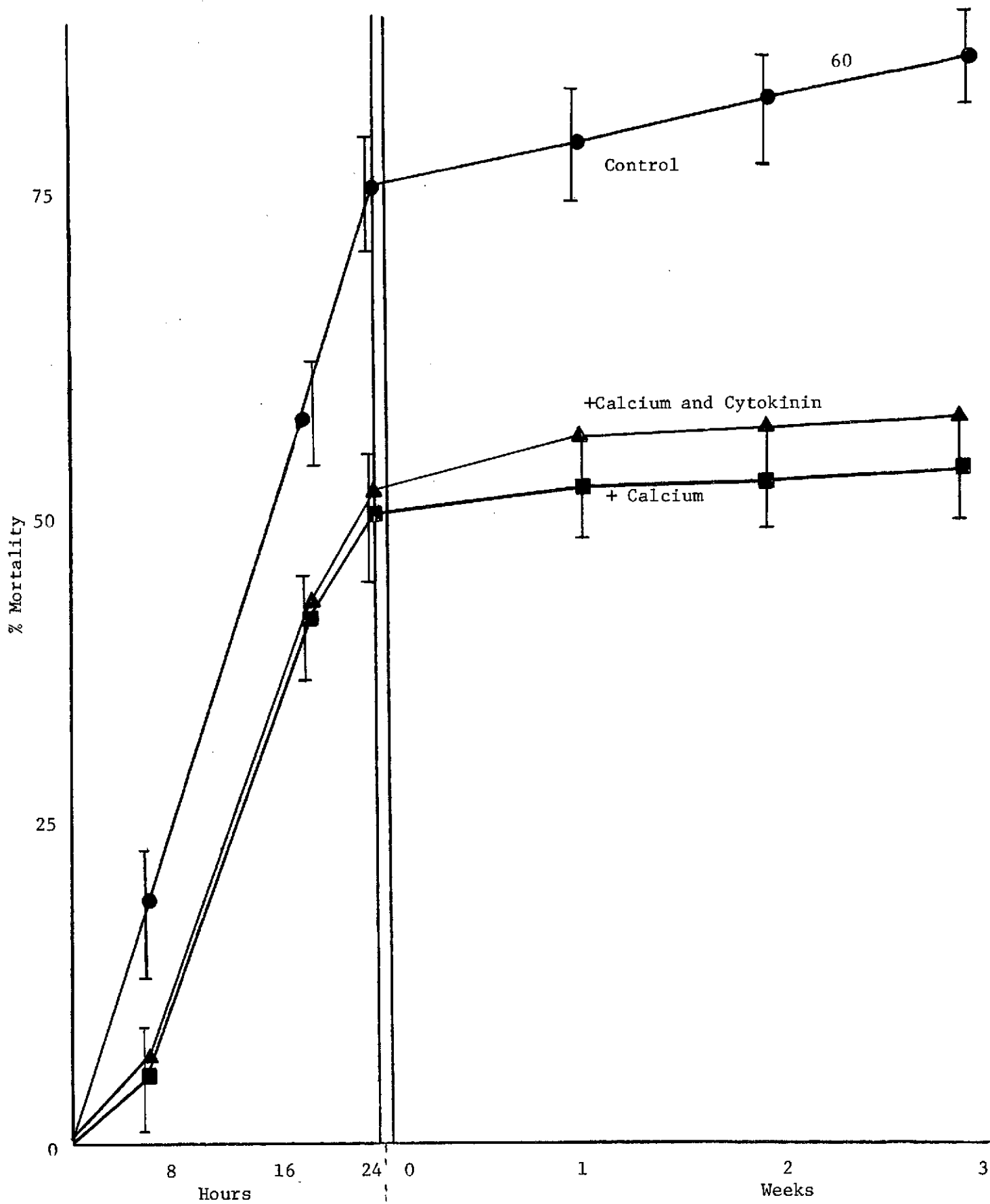


Figure 13. Cold temperature protection of *Axonopus affinis* by calcium.

## SUMMARY

Low temperature endurance in plants is related to a syndrome of membrane deterioration. Mechanisms which permit growth at low temperatures are active at the membrane. The hormone cytokinin is related to this process, but the site and mechanism of its action are unclear. Mineral ions are involved in low temperature endurance mechanisms directly at the membrane. Endurance properties of plants used in this study permit growth in meristematic tissue, at the tip, at 0 C. The calcium ion is strongly implicated in direct protection of membranes from low temperature damage, and it is selectively transported to these tip regions. Stress response in this environment seems to involve plant membranes in three closely related ways: the antagonism of potassium and calcium, the selective movement of calcium to the tip, and the protective effect of the calcium ion.

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## APPENDIX

Differential Equation Set for Circuit in Figure 9

X = voltage across subscripted element

Y = current through subscripted element

$$\text{Nodal} - Y_s = Y_{rfr} = Y_{rsb} + Y_{rur} + Y_{cr}$$

$$Y_{rsb} = Y_{rsa} + Y_{rl} + Y_{cl}$$

$$Y_{rsa} = Y_{rt} + Y_{ct}$$

therefore,

$$Y_s = 1/R_{rfr} X_{rfr} = 1/R_{rsb} X_{rsb} + 1/R_{rur} X_{rur} + C_r X_{cr}$$

$$1/R_{rsb} X_{rsb} = 1/R_{rsa} X_{rsa} + 1/R_l X_{rl} + C_l X_{cl}$$

$$1/R_t X_{rt} + C_t X_{ct} = 1/R_{rsa} X_{rsa}$$

$$\text{Loop} - X_s + X_{rfr} + X_{cr} = 0 \quad X_{cr} = X_{rur}$$

$$X_{rur} + X_{rsb} + X_{cl} = 0 \quad X_{cl} = X_{rl}$$

$$X_{rl} + X_{rsa} + X_{ct} = 0 \quad X_{ct} = X_{rt}$$

therefore,

$$Y_s R_s = R_{rfr} Y_{rfr} + 1/c_r \int Y_{cr} dt \quad 1/c_r \int Y_{cr} dt = R_{rur} Y_{rur}$$

$$R_{rur} Y_{rur} = R_{rsb} Y_{rsb} + 1/c_l \int Y_{cl} dt \quad 1/c_l \int Y_{cl} dt = R_l Y_{rl}$$

$$R_l Y_{rl} = R_{rsa} Y_{rsa} + 1/c_t \int Y_{ct} dt \quad 1/c_t \int Y_{ct} dt = R_t Y_{rt}$$

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